Volatile anesthetics reduce invasion of colorectal cancer cells through down-regulation of matrix metalloproteinase-9

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Abstract: BACKGROUND: Invasion of extracellular matrix is a hallmark of malignant tumors. Clamping maneuvers during cancer surgery reduce blood loss, but trigger reperfusion injury (RI). RI increases cancer recurrence in the reperfused organ through up-regulation of matrix metalloproteinase-9 (MMP-9). Interleukin-8 is an important cytokine in RI promoting accumulation of neutrophils, a major source of MMP-9. Volatile anesthetics were demonstrated to reduce RI. We hypothesized that these anesthetics might attenuate MMP-9 up-regulation and consequently tumor cell invasion in RI. METHODS: Isolated human neutrophils (n = 6) were preconditioned with sevoflurane or desflurane, followed by stimulation with interleukin-8, phorbol myristate acetate, or chemokine CXC-ligand 1 (CXCL1) to differentiate intracellular pathways. MMP-9 release and activity were quantified by enzyme-linked immunosorbent assay and zymography, respectively. CXC-receptor-2 (CXCR2) expression and phosphorylation of extracellular signal-regulated kinases 1/2 were assessed by flow cytometry. The impact of MMP-9 on the invasion of neutrophils and MC-38 colon cancer cells was assessed using Matrigel-coated filters (n = 6). RESULTS: Preconditioning reduced interleukin-8-induced MMP-9-release by 41% (±13, 5%, sevoflurane) and 40% (±13%, desflurane). This was also evident following stimulation of CXCR2 with CXCL1. No impact on phosphorylation of extracellular signal-regulated kinases 1/2 and MMP-9 release was observed with receptor-independent stimulation of protein kinase C with phorbol myristate acetate. Preconditioning reduced transmigration of neutrophils and MC-38 tumor cells to baseline levels. DISCUSSION: Volatile anesthetics impair neutrophil MMP-9 release and interfere with pathways downstream of CXCR2, but upstream of protein kinase C. Through down-regulation of MMP-9, volatile anesthetics decrease Matrigel breakdown and reduce subsequent migration of cancer cells in vitro.

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Volatile Anesthetics Reduce Invasion of Colorectal Cancer Cells through Down-regulation of Matrix Metalloproteinase-9

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ABSTRACT

Background: Invasion of extracellular matrix is a hallmark of malignant tumors. Clamping maneuvers during cancer surgery reduce blood loss, but trigger reperfusion injury (RI). RI increases cancer recurrence in the reperfused organ through up-regulation of matrix metalloproteinase-9 (MMP-9). Interleukin-8 is an important cytokine in RI promoting accumulation of neutrophils, a major source of MMP-9. Volatile anesthetics were demonstrated to reduce RI. We hypothesized that these anesthetics might attenuate MMP-9 up-regulation and consequently tumor cell invasion in RI.

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Discussion: Volatile anesthetics impair neutrophil MMP-9 release and interfere with pathways downstream of CXCR2, but upstream of protein kinase C. Through down-regulation of MMP-9, volatile anesthetics decrease Matrigel breakdown and reduce subsequent migration of cancer cells *in vitro*.

What We Already Know about This Topic

- Matrix metalloproteinase-9 (MMP-9) facilitates tumor invasion and migration by degrading extracellular matrix
- Neutrophil release of MMP-9 through protein kinase C (PKC) activation is increased by interleukin-8 (IL-8) stimulation during postischemia reperfusion
- Potent volatile anesthetics attenuate reperfusion injury and decrease up-regulation of stimulated neutrophil inflammatory adherence molecules

What This Article Tells Us That Is New

- Volatile anesthetic preconditioning reduced MMP-9 release by IL-8-stimulated human neutrophils *in vitro*
- This effect was mediated downstream of the IL-8 receptor and upstream of PKC
- MMP-9 down-regulation reduced mouse colon carcinoma cell migration across simulated extracellular matrix *in vitro*

SURGICAL resection is the primary treatment for patients suffering from colorectal cancer. For higher stages of disease with tumor spread, surgical removal of isolated liver metastases combined with chemotherapy is common and may be curative in selected patients.[1] The 5-yr survival rate of such patients qualifying for curative liver resection is 40–50%, mostly because of recurrent malignancies within the

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liver.[1] This poor outcome might be partially influenced by the hepatic resection itself. First, surgical manipulation leads to the dissemination of cancer cells into the blood.[2,3] Second, clamping maneuvers to anticipate large blood losses are known to result in reperfusion injury of the liver,[4,5] which has been shown to increase both the number and the size of liver metastasis in animal models of colorectal cancer.[4,6]

There is a considerable amount of evidence that matrix metalloproteinase-9 (MMP-9), also referred to as gelatinase B, plays an important role at different steps of malignant tumor growth.[7] MMP-9 belongs to a family of zinc-dependent gelatinases that degrades components of the extracellular matrix.[8] Before invading blood or lymph vessels, malignant tumor cells are obliged to cross multiple physical barriers, such as the extracellular matrix.[9] By degrading the extracellular matrix, MMP-9 promotes this process and facilitates tumor invasion and migration.[7] Several other proteinases are also able to degrade components of the extracellular matrix. However, matrix metalloproteinases appear to be of particular importance with specific members being predominant for certain malignant tumors.[8] MMP-9 was demonstrated to have a strong impact on colon cancer.[7,10,11]

Albeit the fact that tumor cells are able to produce MMP-9, the richest source of MMP-9 in the body are neutrophils. Neutrophils produce MMP-9 constitutively at a low basal rate and dramatically increase MMP-9 release upon stimulation with the proinflammatory cytokine interleukin-8 (IL-8).[12] High amounts of IL-8 are released during the reperfusion phase after ischemia and promote the influx of large numbers of neutrophils into the reperfused organ.[13–15] Volatile anesthetics were demonstrated to attenuate reperfusion injury *in vivo*[16–18] and to decrease the up-regulation of inflammatory adherence molecules of stimulated neutrophils *in vitro*.[19–21]

Therefore, we hypothesized that volatile anesthetics not only attenuate up-regulation of cell surface molecules, but also reduce the release of MMP-9, stored in neutrophil granules. This study further aims at investigating if volatile anesthetic-induced decreased levels of MMP-9 are functionally potent enough to impair degradation of extracellular matrix and subsequent invasion of tumor cells.

Materials and Methods

Isolation of Neutrophils

Citrated venous blood was collected after informed consent from volunteers with no history of illness in the past 14 days. Ethical approval was received from the Ethic Committee of the University Hospital Zurich (Zurich, Switzerland). Red blood cells were lysed and neutrophils were isolated by gradient centrifugation as described before.[22] The remaining cell suspension was layered over Ficoll-Histopaque 1077 (Sigma-Aldrich, St. Louis, MO), centrifuged at 1,500 rpm for 30 min, and resuspended at 2×10^6 neutrophils/ml in Ham's F-12 medium supplemented with 0.5% bovine serum albumin (Invitrogen, Carlsbad, CA). All steps were performed at 4°C to prevent artificial activation of neutrophils. Before each experiment, neutrophils were examined under the light microscope for signs of artificial activation such as clumping, which were absent.

Preconditioning with Volatile Anesthetics

Neutrophil cell suspension was seeded in sterile 96-well plates at 50 μl per well (10^5 neutrophils/well) and placed in humified airtight chambers (Oxoid anaerobic jar; Oxoid AG, Basel, Switzerland). Chambers were flushed with an air/5% CO_2-mixture for 5 min that was optionally augmented with 2.2% of sevoflurane (Sevorane®; Abbott AG, Baar, Switzerland, corresponding vaporizer: Sevotec5®; Abbott AG) or 6% of desflurane (Forene®; Baxter, Zurich, Switzerland; corresponding vaporizer: Tec6, Carbamed; Liebefeld, Switzerland). Control cells were exposed to the air/5% CO_2-mixture only. Volatile anesthetic concentrations were measured by the Ohmeda 5330 Agent Monitor (Abbott AG). Chambers were sealed and kept in an incubator (Bioblock, Ittingen, Switzerland) at 37°C for 45min (preconditioning = exposure to volatile anesthetic before stimulation). The concentrations of the volatile anesthetic were again checked at the end of incubation to preclude insufficient sealing.

Stimulation of Neutrophils

Following preconditioning, neutrophils were removed from the chambers and put in an incubator at 37°C flushed with an air/5% CO_2-mixture. Neutrophils were immediately stimulated with 100 nM human IL-8 (recombinant human IL-8, BD Pharmingen, Franklin Lakes, NJ) for 15 min. Neutrophils exposed to air/5% CO_2-mixture during preconditioning followed by stimulation with phosphate-buffered saline served as negative controls. Stimulation was stopped on ice and supernatants were harvested for further analysis.

For experiments on protein kinase C (PKC)-dependent extracellular signal-regulated kinase (ERK) 1/2 phosphorylation and MMP-9 release, neutrophils were preconditioned (see Preconditioning with Volatile Anesthetics), followed by stimulation with 100 nM phorbol myristate acetate (PMA) for 5 min. Stimulation with the chemokine CXC-ligand 1 (CXCL1) at 100 ng/ml for 15 min was used in experiments to trigger through CXC-receptor 2 (CXCR2) MMP-9-release.

Quantification of Total MMP-9 in Supernatant

Concentration of MMP-9 was determined using an enzyme-linked immunosorbent assay (ELISA), according to manufacturer's directions (R&D systems, Minneapolis, MN). This assay measures the 92 kDa pro-MMP-9 and the 82 kDa active MMP-9.

Measurement of Enzymatic Activity of MMP-9

Activity of MMP-9 in supernatants from IL-8- and PMA-stimulated neutrophils was measured using zymography, as described before.[23] Zymography gels containing gelatin as a substrate for MMP-9 were used. MMP-9 activity was indirectly evaluated by quantification of the degraded amount of

gelatin using densitometry. A control of human recombinant MMP-9 (R&D Systems) was run with every gel together with protein molecular marker (SeeBlue2, Invitrogen) to confirm the MMP-9-band. Data were analyzed with ImageJ (Wayne Rasband, Bethesda, MD).

Flow Cytometric Measurement of Neutrophil Viability

Unstimulated neutrophils were exposed to volatile anesthetics (see Preconditioning with Volatile Anesthetics). Cells were harvested and incubated with the cell impermeable DNA-dye 7-aminoactinomycin (7-AAD, BD Biosciences, Franklin Lakes, NJ) for 20 min at room temperature and analyzed by flow cytometry (FACS Canto II, BD Biosciences). Viable cells were defined as staining negative for 7-AAD.

Flow Cytometric Measurement of PKC-dependent ERK1/2 Phosphorylation

Following preconditioning (see Preconditioning with Volatile Anesthetics), neutrophils were stimulated with 100 nM PMA for 5 min at 37°C. Staining of phosphorylated ERK1/2 was performed as previously described.[24] We used an Alexa488-conjugated mouse antihuman pERK1/2 antibody (Cell Signaling, Danvers, MA) at a final staining concentration of 0.05 μg/ml and the appropriate isotype control.

Flow Cytometric Measurement of CXCR2 Expression

Neutrophils were exposed to volatile anesthetics (see Preconditioning with Volatile Anesthetics). Following exposure for 45 min, they were stained on ice for 30 min with antibodies reactive to IL-8 receptor CXCR2 (mouse antihuman CXCR2 at a final staining concentration of 0.25 μg/ml; BD Biosciences). Appropriate isotype controls were run with every experiment. 7-AAD serving as a dead/live-stain was included in every sample, and only cells negative for 7-AAD were included in the analysis.

Neutrophil Migration Assay with Matrigel

Filters of a 96-well migration plate with 8 μm pores (Millipore, Billerica, MA) were coated with 10 μl of pure Matrigel (BD Biosciences). Matrigel is rich in collagen IV, laminin, and proteoglycans, and is widely used to simulate the extracellular matrix *in vitro* during invasion and migration.[25–27] Neutrophil were seeded in the upper compartment, preconditioned with volatile anesthetics, and stimulated with IL-8 (see Stimulation of Neutrophils). After stimulation for 15 min, 150 μl of Ham's F-12 medium supplemented with 10% fetal calf serum (Invitrogen) was added to the lower compartment and neutrophils were allowed to migrate for 2 h at 37°C. Transmigrated neutrophils were quantified by endogenous β-glucuronidase.[28] For each experiment, outliers that deviated more than one SD from the mean of each experimental group were excluded.

MC-38GFP (Green Fluorescent Protein)/Mouse Neutrophil Comigration Assay with Matrigel

Cell Culture of Mouse Neutrophils. *In vitro* differentiated mouse granulocytes were generated as described before.[29] Briefly, isolated bone marrow cells were transfected with murine stem cell virus vector containing estrogen dependent fusion oncoprotein (E2A-Pbx1-ER) that blocks myeloid differentiation. Conditionally immortalized myeloid progenitors were cultivated in the Roswell Park Memorial Institute medium (Invitrogen) supplemented with granulocyte macrophage colony-stimulating factor and 1 μM β-Estradiol. On removal of estrogen, progenitor clones differentiated to granulocytes and were characterized with staining of CD11b, Gr-1, and F4/80 antibodies.

Mouse colon carcinoma cell line MC-38GFP were grown in Dulbecco's Modified Eagle medium with 10% fetal calf serum.[30] Doubling time of MC-38GFP in culture is approximately 12 h.

Migration. Mouse neutrophils (2×10^5) were seeded on Matrigel-coated 24-well migration plates with 8 μm pores (BD Biosciences) and preconditioned with volatile anesthetics (as described for human granulocytes in Preconditioning with Volatile Anesthetics). Stimulation was performed with 100 ng/ml CXCL1 (recombinant mouse CXCL1, R&D Systems) for 15 min. Starved MC-38GFP cells (2×10^4) were then added to the granulocytes. Medium containing 10% fetal calf serum served as unspecific chemoattractant and was added to the lower compartment. Cells were allowed to migrate for 6 h at 37°C in an incubator free of volatile anesthetics.

Analysis. Nonmigrated cells were removed from the upper chamber with a cotton swab. Filters were fixed with paraformaldehyde and stained with diamidinophenylindole. Sixteen automatically randomized pictures of each sample were taken on a Leica LX microscope (Leica, Buffalo Grove, IL) and GFP/diamidinophenylindole cells counted. Data are given as total number of migrated cells.

Statistical Analysis

All experiments were performed at least three times with blood from different donors. For experiments with n = 6 or more for each condition, normal distribution was assessed using the Kolmogorov–Smirnov-test. Normally distributed values were expressed as mean ± SD and analyzed using one-way ANOVA with a Bonferroni *post hoc* correction to reduce the probability of a type I error. For zymography experiments with n = 3 for each condition, nonparametric testing with Kruskall-Wallis test was performed. Values are expressed as median and interquartiles.

$P < 0.05$ was considered significant. Statistical analyses were calculated with PASW 19 for OSX (SPSS Inc., Chicago, IL). Graphs were made with Graphpad Prism 5 for Macintosh (Graphpad Software, La Jolla, CA).

Results

Isolation of Neutrophils

The isolation procedure yielded neutrophils with a purity of more than 94% with less than 1% of dead cells (defined as 7-AAD+ cells) and less than 2.5% of apoptotic cells (defined as 7-AAD-/Annexin+ cells). No artificial activation of neutrophils occurred.

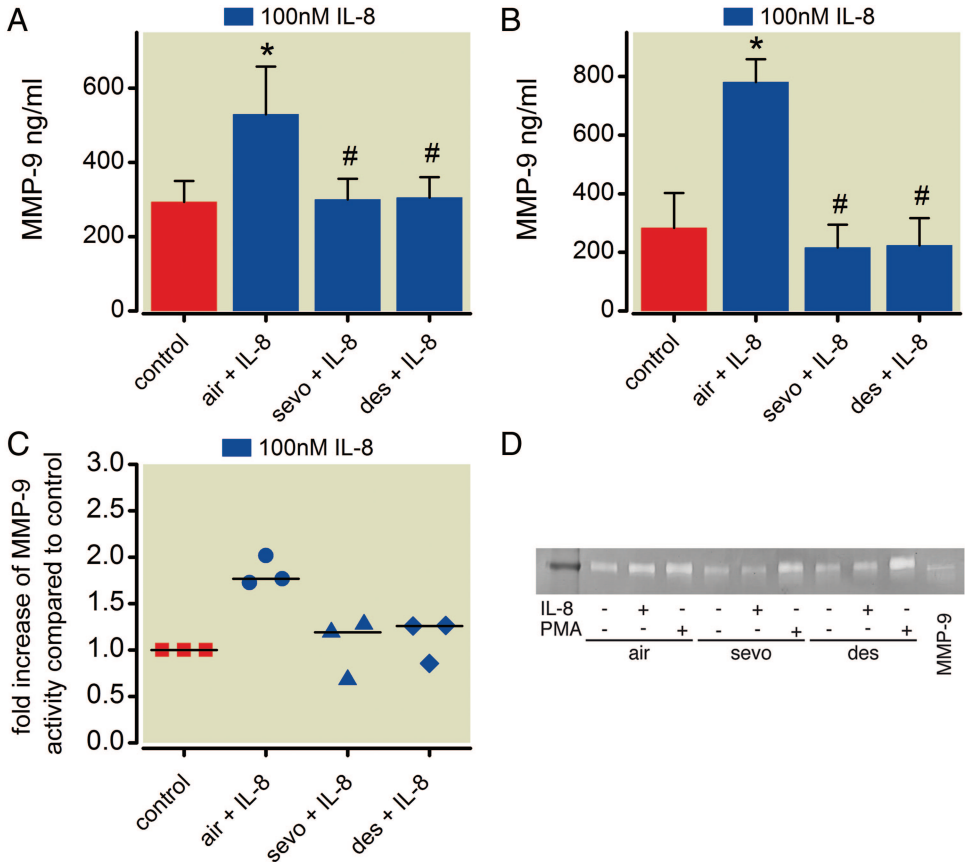

Fig. 1. (*A*) Matrix metalloproteinase-9 (MMP-9) release from neutrophils. Neutrophils were preconditioned with 2.2% sevoflurane (sevo + interleukin-8 [IL-8]), 6% desflurane (des + IL-8), or an air/5% CO_2 mixture (air + IL-8) for 45 min at 37°C. Neutrophils were then moved to an air/5% CO_2-flooded incubator free of volatile anesthetics and stimulated with 100 nM IL-8 for 15 min. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. Control refers to neutrophils that were exposed to air/5%CO_2 during preconditioning and stimulated for 15 min with phosphate-buffered saline instead of IL-8. *$P < 0.01$ for control *versus* air + IL-8, #$P < 0.01$ for air + IL-8 *versus* sevo + IL-8/des + IL-8. (*B*) Preconditioning with reduced volatile concentrations. Neutrophils were preconditioned with 1.1% sevoflurane (sevo + IL-8), 3% of desflurane (des + IL-8), or an air/5% CO_2 mixture (air + IL-8) followed by stimulation with IL-8 for 15 min. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. Control refers to neutrophils exposed to air/5% CO_2 during preconditioning followed by stimulation with phosphate-buffered saline instead of IL-8. *$P < 0.001$ for control *versus* air + IL-8, #$P < 0.001$ for air + IL-8 *versus* sevo + IL-8/des + IL-8. (*C*) Enzymatic activity of MMP-9. Enzymatic activity of MMP-9 in supernatants from neutrophils following preconditioning as described for Fig. 1A and stimulation with IL-8 was assessed by zymography. Data from three separate experiments (n = 3 for each variable) are presented. Control refers to neutrophils exposed to air/5% CO_2 and stimulated for 15 min with phosphate-buffered saline instead of IL-8. $P = 0.083$ for control *versus* air + IL-8/sevo + IL-8/des + IL-8. (*D*) Representative zymography gel. The MMP-9 band was identified by their molecular weight (dark band represents marker at 92 kDa) and human recombinant MMP-9 as loading control (outer right band, MMP-9). PMA = phorbol myristate acetate.

Volatile Anesthetics Reduce Release of Neutrophilic MMP-9

Stimulation of neutrophils under control conditions (air/5% CO_2) with 100 nM IL-8 for 15 min lead to a significant increase in MMP-9 (fig. 1A; control *vs.* air + IL-8, $P = 0.005$). When preconditioned with 2.2% of sevoflurane or 6% of desflurane for 45 min, release of MMP-9 upon stimulation was completely blunted. Compared with IL-8 stimulation with an air/5% CO_2-mixture incubation, preconditioning with volatile anesthetics decreased release of MMP-9 upon IL-8 stimulation by 41% (±13, 5%) and 40% (±13%), respectively (air + IL-8 *vs.* sevo + IL-8, $P = 0.007$, air + IL-8 *vs.* des + IL-8, $P < 0.001$). A comparable effect of preconditioning was also observed with reduced concentrations of volatile anesthetic of 1.1% sevoflurane or 3% desflurane (fig. 1B; air + IL-8 *vs.* sevo + IL-8/des+IL-8, $P < 0.001$).

Enzymatic Activity of MMP-9 Corresponds to MMP-9 Protein Expression

To quantify enzymatic activity of MMP-9, zymography was performed. Enzymatic activity was influenced by preconditioning with volatile anesthetics comparable with the effect on MMP-9 protein (fig. 1C, median and interquartile range; control 1.0 [0], air + IL-8 1.76 [0.29], sevo + IL-8 1.19

[0.6], des + IL-8 1.26 [0.4]; $P = 0.083$). A representative zymography gel is shown in figure 1D.

Changes in Release of MMP-9 Are Not Because of Cytotoxic Effect of Volatile Anesthetics

Neutrophil viability was assessed to exclude a cytotoxic effect of preconditioning with volatile anesthetics. No differences in 7-AAD negative cells were noted with viability rates more than 92% in all groups (data not shown).

PKC-dependent Phosphorylation of ERK1/2 and Release of MMP-9 Is Not Altered by Volatile Anesthetics

Direct stimulation of PKC with PMA induced a strong phosphorylation of ERK1/2 in all groups (fig. 2A; control *vs.* air + PMA/sevo + PMA/des + PMA, $P < 0.001$). Preconditioning with volatile anesthetics did not influence ERK1/2-phosphorylation through PKC (air + PMA *vs.* sevo + PMA, −18.6% [±26.5%], $P = 0.37$; air + PMA *vs.* des + PMA, −19.5% [±17.8%]; $P = 0.27$). No difference was noted between sevoflurane and desflurane preconditioning (sevo + PMA *vs.* des + PMA, $P = 1.0$).

As a consequence, preconditioning did not alter release of MMP-9 from PMA-stimulated neutrophils (fig. 2B, air + PMA *vs.* sevo + PMA, +5.2% [±12.2%]; air + PMA *vs.* des + PMA, +8.8% [±25.6%], $P = 1.0$).

Protection with Volatile Anesthetics Is Also Observed Upon Activation of IL-8 Receptor CXCR2

Stimulation with 100 ng/ml of CXCL1 induced a 51% (±26.2%) increase in MMP-9 release (fig. 3, control *vs.* air + CXCL1, $P = 0.002$). Preconditioning with volatile anesthetics for 45 min before stimulation with CXCL1 significantly reduced MMP-9 release by 29% (±14.8%) and 42% (±10.7%), respectively (air + CXCL1 *vs.* sevo + CXCL1, $P = 0.009$; air + CXCL1 *vs.* des + CXCL1, $P < 0.001$).

Volatile Anesthetics Do Not Alter Expression of the IL-8 Receptor CXCR2

To further assess the role of IL-8 receptor CXCR2 in mediating volatile anesthetic preconditioning, surface expression of CXCR2 was quantified following exposure to volatile anesthetics. Neither sevoflurane nor desflurane altered CXCR2 expression (fig. 4).

Preconditioning Reduces Matrigel Permeability Through MMP-9 Down-regulation

In the current study, higher MMP-9 levels following IL-8 stimulation lead to an increase in permeability of the matrigel layer for neutrophils (fig. 5; control *vs.* air + IL-8, +31.5% [±36.5%], $P < 0.001$). Preconditioning prevented this increase and reduced neutrophil migration to unstimulated levels (air + IL-8 *vs.* sevo + IL-8, $P = 0.011$; air + IL-8 *vs.* des + IL-8, $P < 0.001$).

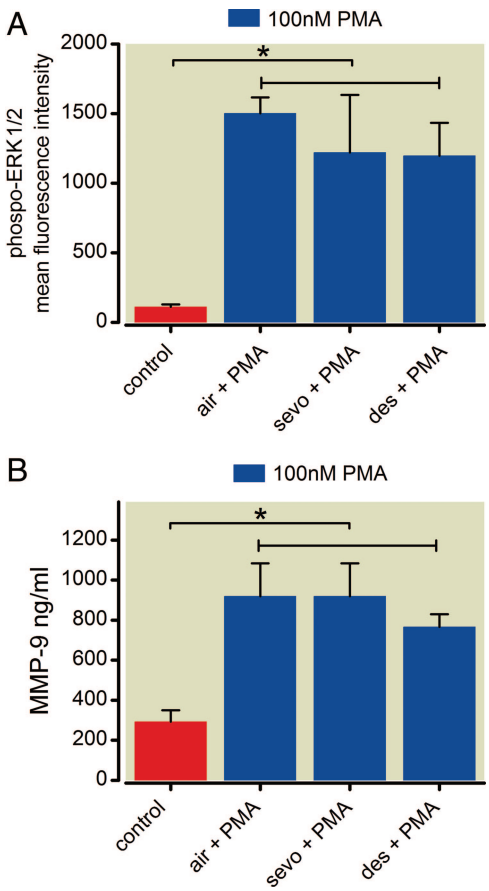

Fig. 2. (*A*) Protein kinase C-dependent phosphorylation of extracellular regulated kinase (ERK)1/2. Neutrophils were preconditioned with volatile anesthetics for 45 min and then stimulated with 100 nM phorbol myristate acetate (PMA) for 5 min in an air/5% CO_2-flooded incubator. Phosphorylation of ERK 1/2 was quantified by flow cytometry. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. *$P < 0.001$ for control *versus* air + PMA/sevo + PMA/des + PMA. (*B*) Protein kinase C-dependent matrix metalloproteinase-9 release. Following preconditioning, neutrophils were stimulated with PMA for 5 min in an air/5% CO_2-flooded incubator. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. *$P < 0.001$ for control *versus* air + PMA/sevo + PMA/des + PMA. Des = desflurane; MMP = matrix metalloproteinase-9; phospho-ERK 1/2 = dependent phosphorylation of extracellular regulated kinase 1/2; PMA = phorbol myristate acetate; Sevo = sevoflurane.

Preconditioning of Neutrophils Reduces Tumor Cell Transmigration

Transmigration through extracellular matrix is required during metastatic seeding. As preconditioning reduced the permeability of Matrigel for neutrophils, the impact on tumor cell transmigration was evaluated. MC-38GFP cells, derived from mouse colon carcinoma, were allowed to migrate in the presence of neutrophils either with or without preconditioning. Stimulation of neutrophils strongly increased tumor cell transmigration (fig. 6; control *vs.* air + CXCL1, $P < 0.001$).

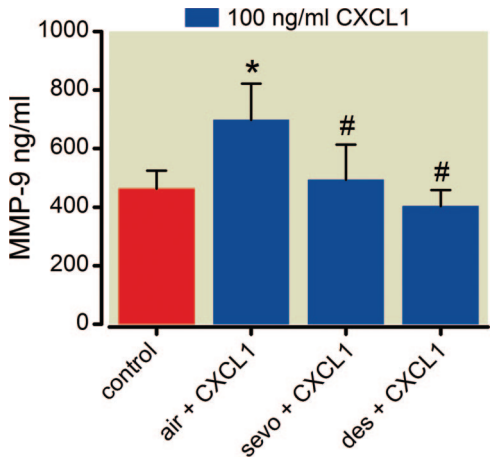

Fig. 3. Stimulation of the interleukin-8 CXC-receptor-2 with CXC-ligand 1 (CXCL1). Neutrophils were preconditioned with 2.2% sevoflurane (sevo + CXCL1), 6% desflurane (des + CXCL1), or an air/5% CO_2 mixture (air + CXCL1), followed by stimulation with 100 nM CXCL1 in an air/5% CO_2-flooded incubator. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. *$P < 0.01$ for control *versus* air + CXCL1, #$P < 0.01$ for air + CXCL1 *versus* sevo + CXCL1/des + CXCL1. CXCL1 = CXC-ligand 1; MMP = matrix metalloproteinase.

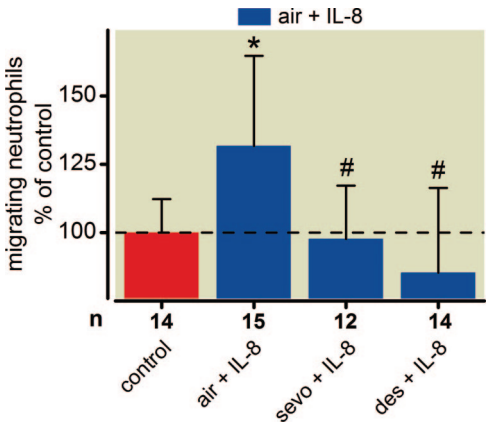

Fig. 5. Matrigel permeability. Neutrophils were preconditioned and stimulated on Matrigel-coated migration filters. Neutrophils were allowed to migrate for 2 h in an incubator free of volatile anesthetics. Data from four separate experiments, each condition repeated in quadruples, are given. Outliers, defined as deviating more than 1 SD from the mean of the group for each separate experiment, were excluded from further analysis. Final number of samples in each variable is given in the figure. Bars show mean and SD. *$P <$ 0.001 for control *versus* air + interleukin-8 (IL-8), #$P < 0.05$ for air + IL-8 *versus* sevo + IL-8/des + IL-8. Des = desflurane; IL-8 = interleukin-8; Sevo = sevoflurane.

Preconditioning of neutrophils reduced the number of transmigrated tumor cells through Matrigel to baseline levels (air + CXCL1 *vs.* sevo + CXCL1/des + CXCL1, $P < 0.001$).

Discussion

Metastatic disease is one of the main factors affecting survival in patients with cancer.[31] MMP-9 was demonstrated to be correlated with tumor invasion and metastatic seeding.[10,11,32] This study provides evidence that preconditioning with sevoflurane or desflurane at clinically used concentrations reduces the release of MMP-9 from human neutrophils in an *in vitro* model of reperfusion injury. The effect by which volatile anesthetics reduce the release of MMP-9 is located downstream of the IL-8 receptor CXCR2, but upstream of PKC. Through down-regulation of MMP-9, preconditioning also reduces the migration of neutrophils and the invasiveness of tumor cells across the extracellular matrix.

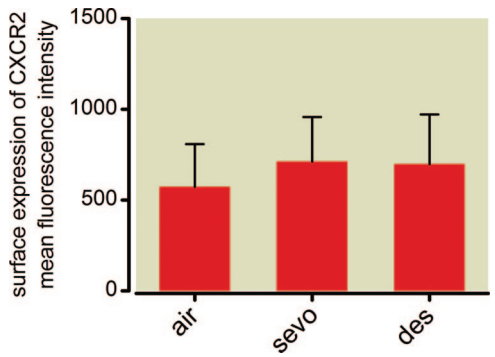

Fig. 4. Interleukin-8 CXC-receptor-2 (CXCR2). Neutrophils were preconditioned with volatile anesthetics for 45 min, followed by staining on ice with anti-CXCR2 antibodies for 30 min. Expression of CXCR2 was analyzed by flow cytometry. Data from three separate experiments, each sample repeated in duplicate, are given (n = 6 for each variable). Bars show mean and SD. Des = desflurane; Sevo = sevoflurane.

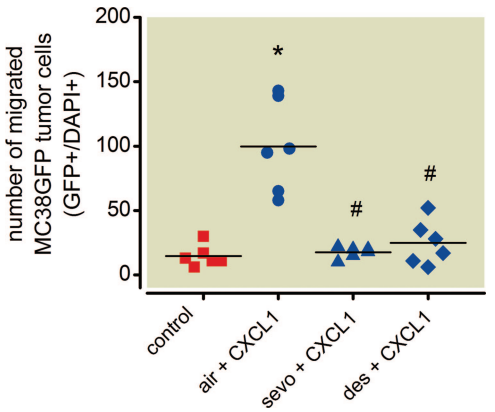

Fig. 6. Invasion of MC-38 green fluorescent protein tumor cells. Mouse neutrophils were preconditioned and stimulated with CXC-ligand 1 (CXCL1). MC-38 green fluorescent protein tumor cells were added and allowed to migrate for 6 h. Scatter plot shows median from six separate migration filters for each condition (n = 6 for each condition). *$P <$0.001 for control *versus* air + CXCL1, #$P < 0.001$ for air + CXCL1 *versus* sevo + CXCL1/des + CXCL1. CXCL1 = CXC-ligand 1; DAPI = diamidinophenylindole; Des = desflurane; MC38GFP = MC-38 green fluorescent protein tumor cells; Sevo = sevoflurane.

The perioperative period is of great interest regarding the risk for recurrence after surgery for colorectal cancer. Only recently, circulating tumor cells measured postoperatively were identified as independent predictors of recurrence of colorectal cancer.[33] To form metastasis, these tumor cells need to extravasate from the bloodstream and migrate through extracellular matrix and basement membrane. MMP-9 promotes this process, first through direct degradation of extracellular matrix and then through the formation of premetastatic niches.[34] Reducing MMP-9-release through preconditioning with volatile anesthetics might therefore come just in time before circulating tumor cells seed to the target organ.

This work demonstrates that preconditioning with volatile anesthetics reduces the release of MMP-9 from IL-8-stimulated neutrophils. The currently commercially available ELISA kits do not distinguish between the inactive pro-MMP-9 and its active form, but rather measure total MMP-9. MMP-9 concentrations in the current study correlated to MMP-9 activity as evidenced by zymography, further validating the ELISA results.

Volatile anesthetics were often demonstrated to attenuate neutrophil activation *in vitro*[19,21] and *in vivo*.[17,18,35] The observed attenuation of MMP-9 is unique since in contrast to neutrophil surface molecules involved in activation and adhesion such as integrins and selectins, MMP-9 is regulated at the level of tertiary granule release, a subgroup of the peroxidase-negative granules.[36,37] IL-8 stimulates the release of MMP-9 through activation of PKC, which in turn phosphorylates ERK1/2.[12] The direct stimulation of PKC with PMA led to a strong phosphorylation of ERK1/2 in our setting. However, preconditioning did not affect this process. Similarly, MMP-9 release from PMA-stimulated neutrophils was not altered by preconditioning with volatile anesthetics. We therefore conclude that volatile anesthetic preconditioning does not directly interfere with tertiary granule release, but rather affects stimulatory pathways upstream of PKC.

Most neutrophil responses to IL-8, such as cell adhesion or release of primary granules, are triggered through activation of both IL-8 receptors CXCR1 and CXCR2.

In contrast, tertiary granule release was shown to rely exclusively on activation of CXCR2.[12] CXCL1 and IL-8 both belong to the family of CXC-cytokines. However, CXCL1 binds to CXCR2 only.[38] Accordingly, we found that stimulation of CXCR2 with CXCL1 led to an increase in MMP-9. Similarly to the combined activation of CXCR1 and CXCR2 with IL-8, MMP-9 release from CXCL1-stimulated neutrophils was inhibited by the application of volatile anesthetics. This suggests that CXCR1 is not necessary to mediate the effect of preconditioning with volatile anesthetics on tertiary granule release.

Only surface-bound CXCR2 is able to form ligand-receptor complexes with IL-8 or CXCL1, resulting in internalization of the complex and downstream signaling. But internalization is also thought to act as a stop mechanism to reduce neutrophil activity once the neutrophil reached the site of inflammation.[39] No differences in CXCR2 expression following volatile anesthetic preconditioning were found in the current study. This argues against a direct interaction between the volatile anesthetic and CXCR2 that might have triggered internalization of the receptor.

Downstream events following stimulation of CXCR2 with IL-8 include the rapid increase in intracellular calcium.[40] Intracellular calcium triggers a wide range of neutrophil responses in inflammation that are influenced by volatile anesthetics, such as oxidative burst[20,41] and integrin up-regulation and adhesion.[19,42] In contrast to those, the release of MMP-9 was demonstrated to be independent from changes in intracellular calcium concentration.[12] This suggests that preconditioning with volatile anesthetics regarding tertiary granule release is mediated through calcium-independent pathways.

Finally, the biologic relevance of the observed differences in MMP-9 release on the integrity of the extracellular matrix was investigated. Matrigel is derived from Engelbreth-Holm-Swarm sarcoma and consists mostly of collagen IV, laminin, and proteoglycans.[26] It is widely used as an equivalent for extracellular matrix when studying tumor invasion.[25,27] MMP-9 degrades components of Matrigel, and the migration of invasive cells through Matrigel has previously been shown to rely on MMP-9 expression.[43,44] The current study demonstrated that down-regulation of MMP-9 by preconditioning with volatile anesthetics reduced the number of neutrophils that were able to cross the extracellular matrix.

It seems reasonable that invasive tumor cells as well should be influenced by different extents of extracellular matrix degradation. This is supported by Sun *et al.*, who demonstrated that the migration of oral squamous cell carcinoma cells through Matrigel is almost completely dependent on the tumors' ability to secrete MMP-9, and failing to do so suppresses the transmigration process.[45] Therefore, this investigation focused on the MC38 cell line that is derived from mouse colon cancer, as metastatic seeding for these tumor cells was demonstrated to be dependent on MMP-9 expression.[7] Neutrophil preconditioning hindered the subsequent migration process and reduced the number of tumor cells that successfully crossed the extracellular matrix barrier. This experimental setup clearly underlines the functional consequence of decreased neutrophilic MMP-9 secretion upon exposure to volatile anesthetics *in vitro*.

The use of Matrigel is currently the most common model for studying tumor invasion *in vitro*. This is mostly because of the lack of tissue preparation heterogeneity that is observed with other substrates such as amnion or chorioallantoic membranes, resulting in difficult reproducibility of these models. Although routinely used, there might be additional, unknown components of extracellular matrix that are lacking in the Matrigel preparation. Therefore, further *in vivo* studies

are clearly warranted before making clinical conclusions of Matrigel-derived invasion studies.

In vitro studies on volatile anesthetics face two problems: first, standard concentrations for volatile anesthetics are usually defined according to the patient's response to the anesthetic, and might not be simply transferable to cell experiments. In the current study it was decided to adhere to the clinically most relevant concentrations of 0.5 or 1 minimum alveolar concentration, as used by many other authors as well.[19,20] Second, the experimental setup of exposure to volatile anesthetics might influence the absolute concentration of anesthetic that can reach the cells. However, the current method of cell exposure to volatile anesthetics is a well-established procedure in our lab that has yielded well reproducible results.[46–49]

In conclusion, this study demonstrates that volatile anesthetics attenuate IL-8-induced MMP-9 release from tertiary granules downstream of CXCR2 and reduce the migratory behavior of neutrophils in Matrigel. Consequently, the invasive behavior of tumor cells facilitated through neutrophils has been abrogated. Although these first *in vitro* results are promising, further studies are warranted to evaluate the role of volatile anesthetics in tumor cell migration and spreading *in vivo*.

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